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(57) Abstract

Transgenic plants with increased amounts of high molecular weight glutenin subunits (HMW-GS) have improved breadmaking capability. A specific example of wheat (cv Bob White) transformed to express HMW-GS 1Ax1 is provided. In addition, advantageous uses of the 1Ax1 promoter are described.

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DESCRIPTION

TRANSFORMED WHEAT HAVING IMPROVED BREADMAKING CHARACTERISTICS

Field of the Invention

This invention pertains to the field of genetically transformed Graminae, particularly transformed wheat having improved breadmaking characteristics.

Background of the Invention

The unique breadmaking characteristic of wheat flour is closely related to the elasticity and extensibility of the gluten proteins stored in the starchy endosperm, particularly the high molecular weight glutenin subunits (HMW-GS) which are important in determining gluten and dough elasticity. The quality of wheat cultivars depends on the number and composition of the HMW-GS present.

Prolamins are a novel group of storage proteins found in the endosperm of cereal grains (Shewry, 1995). The prolamins of wheat are divided into two groups, gliadins and glutenins. Together, they form gluten, a continuous proteinaceous network, during the mixing of wheat flour with water to make dough. The gluten proteins are the largest protein molecules found in nature (Wrigley, 1996). The elasticity (strength) and extensibility (viscosity) of the dough, critical for breadmaking, are closely related to glutenins and gliadins, respectively. These unique properties of wheat gluten, not found in the storage proteins of other cereals, are likely related to the enormous size of the glutenin polymers which have relative molecular masses ranging into the tens of millions (Wrigley, *supra*). Low (weak) gluten elasticity is responsible for the poor breadmaking qualities of wheat cultivars which otherwise have desirable agronomic properties. In such instances the mixing of flour from different cultivars is required in order to produce a blend suitable for breadmaking. Extensive biochemical and genetic investigations have shown that the breadmaking quality of wheat flour is determined particularly by the HMW-GS group of proteins. The HMW-GS are subdivided into high M_r x-type and low M_r y-type subunits. Two genes which are inherited as tightly linked pairs, encoding an x-type and a y-type subunit, are present on the 1A, 1B, and 1D chromosomes of

hexaploid bread wheat (Payne, 1987). All cultivars of wheat, therefore, contain six HMW-GS genes, but only three, four, or five subunits are present, because some of the genes are silent (the 1Ay gene is silent in all bread wheat varieties). The number and composition of HMW-GS present in a cultivar are closely related to the quality of its gluten. HMW-GS may represent up to 10% of the total seed protein, as each HMW-GS accounts for about 2% of the total extractable protein (Seilmeier *et al.*, 1991; Halford *et al.*, 1992). However, the close linkage of HMW-GS genes makes it difficult to manipulate them by traditional breeding methods (Flavell *et al.*, 1989). Recent success in the transformation of wheat (Vasil, 1994), therefore, has provided an opportunity to try to improve the gluten quality of wheat by introducing additional copies of HMW-GS genes (Flavell *et al.*, 1989; Shewry *et al.*, 1995). Seeds of transgenic wheat (cv Bob White) containing a hybrid HMW-GS Dy10:Dx5 gene construct have just recently been shown to accumulate the hybrid HMW-GS to levels similar to those of the endogenous HMW-GS genes. Five HMW-GS–Ax2*, Bx7, By9, Dx5, and Dy10–are present in Bob White endosperm (Blechl and Anderson, 1996). The use of the hybrid gene construct was, therefore, necessary to discriminate between the native proteins encoded by the Dx5 and Dy10 genes, and the hybrid HMW-GS formed by the introduced Dy10 and Dx5 genes.

There exists a continuing need for wheat with improved breadmaking quality, and methods for creating such wheat. Therefore, it would be desirable to obtain wheat improved by transformation with heterologous HMW-GS genes which are expressed to yield improved breadmaking quality.

Brief Summary of the Invention

This invention is methods for producing wheat with improved breadmaking characteristics by transforming wheat with heterologous HMW-GS genes. The subject invention is exemplified by the introduction of the HMW-GS 1Ax1 gene into the Bob White cultivar of wheat *(Triticum aestivum* L.), a cultivar in which the 1Ax1 gene is not present in nature, by the biolistic bombardment of cultured immature embryos. The 1Ax1 gene is known to be associated with good breadmaking quality but is not present in many cultivars (Halford *et al.*, 1992; Payne *et al.*, 1979), including Bob White (Blechl and Anderson, 1996). Of the 21 independent transformed lines selected, 20 expressed

the selectable *bar* gene, and nine the 1Ax1 gene. The amount of HMW-GS 1Ax1 protein produced in the different transgenic lines varied from 0.6 to 2.3% of the total protein, resulting in up to 71% increase in total HMW-GS proteins. The transgenic plants were normal, fertile, and showed Mendelian segregation of the transgenes. The accumulation of HMW-GS 1Ax1 was consistent and stable up to the R3 seed generation. This is the first time that anyone has created wheat in which more than five HMW-GS genes are expressed. Surprisingly, it has been discovered that additional heterologous genes above and beyond the five which are naturally expressed will express without silencing native gene expression. Accordingly, as exemplified herein, the subject invention enables those skilled in the art to predictably manipulate both the quantity and quality of HMW-GS by transforming wheat with heterologous HMW-GS genes of the artisan's choice which influence the breadmaking quality of wheat.

Brief Description of the Drawings

Figure 1. SDS-PAGE of proteins from single R2 seeds of wheat cultivar Bob White transformed with the gene encoding HMW-GS 1Ax1. The bar indicates the location of HMW-GS subunits; the arrows mark the position of subunit 1Ax1. Numbers at the top correspond to the nine individual transformed lines expressing the 1Ax1 gene. HMW-GS 1Ax1 is not present in nontransformed Bob White (lane c). Proteins smaller than 46 kDa are not shown. Protein molecular weight standards are shown on the right.

Figure 2. SDS-PAGE of proteins from single seeds of line 159 (highest expresser) and line 235 (lowest expresser) showing uniformity of accumulation of HMW-GA 1Ax1 in R1, R2, and R3 seed. HMW-GS 1Ax1 is not present in nontransformed Bob White, lane C. Protein molecular weight standards are shown on the left.

Figure 3. SDS-PAGE of proteins from seven single seeds of line 29, homozygous for HMW-GS 1Ax1 gene expression in R2. The bar indicates the location of HMW-GS; the position of subunit 1Ax1 is shown by the arrow. HMW-GS 1Ax1 is not present in nontransformed Bob White (second lane from left). Protein molecular weight standards are shown in the left lane, as in Figure 2.

Figure 4. Southern blot of genomic DNA (20 μg) of lines 28, 31, 87, 85, 25, 30, 51, and 62, and a non-transformed control plant (nc), restricted with *Xba*I and hybridized

with the 1Ax1 probe. The sizes of the two hybridizing bands originating from endogenous HMW-GS genes are indicated in kb (right).

Figure 5. Southern blot of genomic DNA of lines 235, 233, 217, and 159, a nontransformed control plant (nc), and DNA of plasmid pHMW1Ax1 (pc), were restricted with *Xba*I (right) or left undigested (left), and hybridized with the 1Ax1 probe; 10 μg DNA for undigested samples, and 25 μg DNA for digested samples. The sizes of the two hybridizing bands originating from endogenous HMW-GS genes are indicated in kb (right).

Detailed Disclosure of the Invention

The quantity of HMW-GS 1Ax1 present in the endosperm is positively correlated with dough elasticity/strength(Halford *et al.*, 1992; Branlard, 1987). The manipulation of HMW-GS genes through traditional breeding, though possible, has been shown to be unpredictable, difficult, and complicated because of their close linkage (Flavell *et al.*, 1989). The subject invention enables this barrier to the improvement of breadmaking quality of wheat to be overcome by the introduction and expression of additional HMW-GS genes by genetic transformation resulting in qualitative and quantitative changes in HMW-GS. The 1Ax1 gene is not present in the Bob White cultivar of wheat used as an example herein, making it possible to detect its transgenic expression in wheat by SDS-PAGE as a novel additional band, which is absent in the nontransformed control. A majority of the nine independently transformed lines expressing the HMW-GS 1Ax1 gene in R1 showed similar levels of expression in R2 seed extracts (Table 2). Densitometric analysis (Table 2) confirmed that the differences in the relative levels of HMW-GS accumulation between individual transgenic lines, including the amounts of HMW-GS 1Ax1, were stable through three generations. The *bar* and 1Ax1 genes cosegregated in all the eight lines in which they were expressed. This indicates that integration occurred at a single locus in all the lines expressing 1Ax1 and *bar*, both of which were inherited in a Mendelian fashion in all but two lines (25 and 87). Integration of transgenes at single as well as multiple loci in the genome of cereal species has been described (Christou *et al.*, 1989; Spencer *et al.*, 1992; Srivastava *et al.*, 1996). Southern analysis of eight of the nine lines expressing HMW-GS 1Ax1 suggests that the 1Ax1 transgene is integrated in multiple copies in all the lines (Figures 4, 5). Thus far, eight

lines have been identified as homozygous in R2. The expression of the transgene was maintained in successive generations in all lines. The amount of transgenic protein produced varied depending on the individual line, from 0.6 to 2.3% of the total extracted protein. The high level of expression of the introduced 1Ax1 gene, and its stability through at least three generations, suggests that the native HMW-GS gene promoter can be used effectively for the expression of transgenic proteins in the endosperm tissue of wheat and other cereals. Accordingly, use of the 1Ax1 promoter to drive expression of heterologous DNA segments encoding proteins is within the scope of the subject invention. Although scanning densitometry of SDS-PAGE does not provide precise quantitative data, it is nevertheless useful in assessing the effect of introduced HMW-GS genes. The methods taught herein resulted in an increase of up to 71% in total HMW-GS after introduction of the 1Ax1 gene. Surprisingly, the results demonstrate that in most of the lines the accumulation of the transgenic subunit 1Ax1 was not at the expense of the other HMW-GS (although lines 85 and 233 showed only a moderate level of 1Ax1 expression, it was nonetheless at the expense of other HMW-GS, so that the total HMW-GS level was in the range of the nontransformed control).

The subject invention concerns methods for enhancing and increasing the breadmaking characteristics of plants, such as wheat, through the transformation of the plants with genes encoding HMW-GS polypeptides. The subject invention also concerns the transformed and transgenic plants, plant material, and seeds having HMW-GS transgenes, or fragments or variants thereof. In a preferred embodiment, multiple copies of the HMW-GS transgene are integrated into the genome and expressed in the plant cell. The subject invention also encompasses bread and the like prepared from plants and seeds of the present invention.

It is therefore clear that, according to these teachings, the skilled artisan is enabled to increase the total number of HMW-GS genes, and thus the amount of HMW-GS accumulated, resulting in improved breadmaking quality. Further, the subject invention provides the opportunity to routinely manipulate the composition of HMW-GS, and thus its effect on breadmaking quality, by the introduction into wheat and other plants/cereals of genes mutated by means well known in the art to cause alterations in the structure of HMW-GS. In addition, HMW-GS genes can be altered by means well known in the art to add nucleotides (by insertion at restriction enzyme sites, for example) or to

remove nucleotides (by use of *Bal*31 exonuclease, for example) to yield a variant or fragment which encodes a protein according to the teachings herein.

Thus, by following the teachings herein, the skilled artisan is provided the means and expectation that the HMW-GS 1Ax1 gene, or any other HMW-GS gene associated with good breadmaking quality, can be stably integrated, expressed, and inherited as a single dominant locus in the wheat genome following Mendelian inheritance. The following examples specifically show that, under the control of its native HMW-GS promoter, substantial amounts of HMW-GS 1Ax1, novel for the Bob White cultivar of wheat, are produced. These examples illustrate that one of ordinary skill in the art can predictably and routinely alter the composition of the wheat endosperm, and hence its suitability for human and industrial use, by the introduction of relevant HMW-GS genes by transformation according to the teachings herein. Similarly, the composition of maize, rice, and other cereals can be altered as taught herein and according to known transformation and selection techniques. It should be understood that methods of transformation other than the method specifically exemplified herein can be used in the methods of the subject invention and are contemplated within the scope of the invention.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. The methods disclosed in each of the cited references are incorporated herein by reference, and in particular where noted.

Example 1 – Wheat Transformation and Plasmids

Transformation was carried out by bombardment of immature embryos of *Triticum aestivum* L. (cv Bob White) in a biolistic particle acceleration device (PDS1000/He, Bio-Rad) as described previously (Taylor *et al.*, 1993; Vasil *et al.*, 1993; Altpeter *et al.*, 1996; methods of which are incorporated herein by reference). The plasmids pAHC25 and pHMW1Ax1 were mixed in approximately 1:1 molar ratio (5 µl of each DNA) for cotransformation. The plasmid pAHC25 contains the selectable *bar* gene and the GUS reporter gene (*uidA*), both under the control of the maize ubiquitin promoter (Vasil *et al.*, 1993; Altpeter *et al.*, 1996; Christensen and Quail, 1996). The

plasmid pHMW1Ax1 contains the HMW-GS 1Ax1 gene of wheat whose expression is driven by its own endosperm specific promoter (Halford *et al.*, 1992). Transgenic lines were selected on a bialaphos containing medium as described (Altpeter *et al.*, 1996). A total of 21 independent transgenic wheat lines (20 expressing PAT) were obtained from 7650 embryos (in nine experiments), under suboptimal bombardment and selection conditions, giving an overall transformation frequency of 0.3%.

Example 2 – Cotransformation and Expression of HMW-GS 1Ax1 and *bar*

Immature embryos of wheat cultivar Bob White were cotransformed with *pAHC25* and pHMW1Ax1. Twenty independent transformed lines were identified based on determination of phosphinothricin acetyl transferase (PAT) activity. An additional line (line 85) was identified by Southern analysis (both the *bar* and 1Ax1 genes were present) in a separate experiment, carried out on 12 plants that survived selection on bialaphos but showed no PAT activity. Plants were transferred to soil in less than three months after culture initiation. Each of the 21 transformed lines was fully fertile and produced R1 seed. Total proteins were extracted individually from eight mature R1 seeds of each line and analyzed by SDS-PAGE for the accumulation of the transgenic HMW-GS 1Ax1. As shown in Figures 1-3, HMW-GS 1Ax1 protein is not present in control Bob White seeds (control lane). Therefore, the presence of the transgenic 1Ax1 subunit was clearly distinguishable in nine lines (two of these are shown in Figure 2), with the protein banding at *ca.* 126 kDa relative to standard molecular weight markers. Of the 20 lines expressing PAT and cotransformed with pHMW1Ax1, eight also expressed the 1Ax1 transgene, giving a coexpression frequency of 40%.

Example 3 – PAT Assays

Following selection, primary transformants were identified by determination of PAT activity in leaf extracts by silica gel thin layer chromatography (Spencer *et al.*, 1990, methods incorporated herein by reference), except that 2.0 µl of [^{14}C]acetyl-CoA 43.2 mCi/mmol (Sigma) was used as label. The products of the reaction corresponding to 25 µg of total protein were used from each sample.

Example 4 – Protein Analysis

Protein extracts were prepared by grinding mature dry seeds individually with a mortar and pestle. Ten to fourteen mg of the resultant flour from each seed was vortexed with 200 µl sample buffer (2% SDS, 5% β-mercaptoethanol, 0.001% Pyronin Y, 10% glycerol, 0.063 M Tris HCl pH 6.8) for 2 minutes and incubated for 2 hours on a rotary shaker at 250 rpm. The extracts were centrifuged (10 minutes, 14,000 rpm) and the supernatant boiled for 5 minutes to denature the protein. The proteins were separated by SDS-PAGE (Laemmli, 1970); 20 to 30 µl of each sample was loaded in 13 cm gels containing 10% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide and run until the dye front had reached the bottom of the gel, so that the total extracted protein remained on the gel and the 1Ax1 band was resolved from the rest of the HMW-GS which were not completely separated from each other. The gels were first fixed in the staining solution without dye for 0.5 to 1 hour and then stained in Coomassie Brilliant Blue R-250 for 4 to 6 hours (Neuhoff *et al.*, 1988). Protein bands were visualized by destaining in an aqueous solution of 5% methanol and 7% acetic acid (vol/vol) until a clear background was obtained. Gels were stored in a 7% aqueous acetic acid solution (vol/vol). Stained gels were scanned using an Alpha Innotech (San Leandro, CA) IS-1000 Digital Imaging System. Lane and peak values were corrected by interband background subtraction. Background intensity was determined for each individual lane from the top of each HMW-GS 1Ax1 band at approximately 140 kDa. The amount of HMW-GS 1Ax1 present was calculated relative to the corrected lane value or the corrected HMW-GS value. To calculate the total HMW-GS level, the protein contents of each lane were normalized.

Example 5 – DNA Analysis

Genomic DNA was isolated from the leaves of PAT-positive plants by the CTAB method (Lassner *et al.*, 1989, methods incorporated herein by reference). Purified DNA (20 to 25 µg) was digested with *Xba*I, electrophoresed in 0.8% agarose gel, and blotted on Hybond-N membrane (Amersham). The probe for hybridization consisted of the 2.2 kb fragment from the coding region of the HMW-GS 1Ax1 gene, derived after an *Eco*RI and *Hin*dIII digest of pHMW1Ax1, using the random primer labelling kit (GIBCO-BRL).

Hybridization was performed at 65°C for 24 hours, and signals were visualized by autoradiography.

Example 6 – Segregation Analysis

To determine the segregation ratios of transgene *bar* in the R1 generation, 20 mature embryos from each of the 21 transgenic lines were germinated on a medium supplemented with bialaphos: half strength MS-salts and vitamins (Murashige and Skoog, 1962; methods incorporated herein by reference) supplemented with 15 g/l sucrose, 2.5 g/l gelrite, and 3 mg/l bialaphos (added filter sterilized after autoclaving), pH 5.8 (B3 medium). The expression of the unselected 1Ax1 transgene was assessed in each of the successive generations and only the lines expressing the gene were carried on to the next generation. Lines homozygous for *bar* were identified from R2 seeds, by testing the germinability of 20 embryos from up to 12 R1 plants of all HMW-GS 1Ax1 accumulating lines on B3 medium. Ten seeds of each homozygous *bar* line were analyzed individually by SDS-PAGE for HMW-GS 1Ax1 to determine if cosegregation had occurred.

Example 7 – Segregation Analysis, Stability of Coexpression, and Level of HMW- GS Accumulation

Mature embryos excised from 20 R1 and 120-240 R2 seeds of each line (6 to 12 plants/line) were germinated on the bialaphos containing B3 medium, to study segregation and expression of the transgene *bar* in successive generations, and to identify plants homozygous for *bar*. Germination frequencies of 16 lines did not differ significantly from Mendelian segregation for a single integration site; lines *25* and 87 did not show Mendelian segregation (Table 1). PAT expression was lost in R1 plants of lines 62 and 228. Line 85, that carried but did not express the *bar* gene in R0, did not germinate on BS medium. Thus far, seven lines homozygous for PAT expression have been identified in the R2 generation. Coexpression/segregationof the 1Ax1 gene in these seven lines has been confirmed by SDS-PAGE analysis. Line 85, although not showing PAT activity, was found to accumulate HMW-GS 1Ax1; its homozygous progeny has also been identified. In two of the lines (line 87 and 235), an extra polypeptide (195 kDa and 156 kDa, respectively) of unknown origin that migrated slower than the transgenic

HMW-GS 1Ax1, was seen (Figures 1, 2); this polypeptide was also present in subsequent generations. Figure 1 (lanes 28-235) illustrates the differences in the level of accumulation of HMW-GS 1Ax1 between nine different lines. Densitometric scans of gels showed that the amount of HMW-GS 1Ax1 protein produced in the different lines varied from 0.6 to 2.3% of the total protein (Table 2). In the lines (28, 87, 159) showing the strongest expression, 32 to 40% of the total HMW-GS was comprised of the transgenic HMW-GS 1Ax1, without any decrease in the native HMW-GS, resulting in a 1.53- to 1.71-fold higher level of HMW-GS in R1 and R2 seeds, as well as in R3 seeds of line 159, compared to the nontransformed control (Table 2). The accumulation of HMW-GS 1Ax1 within the homozygous lines was uniform (Figure 3). The differences in the relative amounts of HMW-GS 1Ax1 between different lines were largely consistent over successive generations.

Table 1. Germination ratios of mature embryos from R1 seeds on B3 medium (3 mg/l bialaphos)

Transgenic line number	Germinated embryos	Nongerminated embryos	Germination ratio (%)
25	11	9	55
28	16	4	80[a]
29	16	4	80[a]
30	16	4	80[a]
31	15	5	75[a]
51	13	7	65[a]
62	0	20	0
70	16	4	80[a]
85	0	20	0
87	4	16	25
134	17	3	85[a]
136	15	5	75[a]
138	14	6	70[a]
153	15	5	75[a]
159	16	4	80[a]
213	14	6	70[a]
217	16	4	80[a]
228	0	20	0
229	17	3	85[a]
233	14	6	70[a]
235	15	5	75[a]

[a]Analysis using the χ^2 test indicated that segregation ratios of R1 mature embryos from these lines were not significantly different from 3:1 (at a = 0.005).

Table 2. Densitometric analysis of HMW-GS 1Ax1 gene expression in SDS-PAGE gels of total extractable proteins from R1, R2, and R3 seeds of individual wheat lines.

Line No.	Transgenic HMW-GS 1Ax1/total extracted protein (%)[1]			Transgenic HMW-GS 1Ax1/total HMW-GS (%)[2]			Total HMW-GS as % of the control[3]		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
28	1.9	2.0	n.a.	32	34	n.a.	161	171	n.a.
29	1.3	1.0	n.a.	23	17	n.a.	132	159	n.a.
31	0.9	1.2	n.a.	23	22	n.a.	106	160	n.a.
85	0.6	0.6	n.a.	19	18	n.a.	92	104	n.a.
87	2.0	1.8	n.a.	35	32	n.a.	159	169	n.a.
159	2.0	2.2	2.3	36	39	40	153	168	165
217	1.4	0.9	n.a.	29	21	n.a.	135	114	n.a.
233	1.2	0.8	n.a.	29	24	n.a.	114	88	n.a.
235	0.6	0.6	0.6	14	16	15	116	110	114

The values given for R2 and R3 generations are averages of two gels, shown in Figures 1 and 2.
[1,2]The transgenic HMW-GS 1Ax1 peak was divided by the total value for the whole lane [1], or the sum of all HMW-GS peaks of the lane [2].
[3]The sum of all HMW-GS peak values divided by the value for the control lane (Bob White nontransformed) after normalizing the amount of protein. All values shown are after background correction of the individual HMW-GS 1Ax1, HMW-GS, or total protein peaks.
n.a. = not available at the time of analysis.

Example 8 – Southern Analysis

Southern blots of control nontransformed genomic DNA, digested with *Xba*I, showed two cross-reacting bands of 7.0 and 9.3 kb, after hybridization with a probe made from either the full length or the smaller coding region of the pHMW1Ax1 plasmid (control lanes of Figures 4, 5). The enzyme *Xba*I was used for DNA digestion because there is only one *Xba*I restriction site, outside the coding sequence, in pHMW1Ax1. Southern blots were made of genomic DNA from 12 transgenic lines (eight lines expressing 1Ax1, and four lines expressing only the *bar* gene but cotransformed with the plasmid pHMW1Ax1). After digestion with *Xba*I, six lines (28, 31, 85, 87, 233, 235) of the eight lines expressing 1Ax1 showed clear individual patterns of integration (Figures 4, 5), and the presence of multiple copies with irregular insertion and/or truncation of the plasmid. Two lines expressing 1Ax1 (159 and 217) showed an intense 7.0 kb band that comigrated with the endogenous band, but was much more intense than the second endogenous band of 9.3 kb. This suggests the presence of multiple copies in tandem concatemeric arrays. Among lines not expressing 1Ax1, line 25 clearly showed a pattern

of integration that was similar to those of expressing lines 28 and 31. The transgene was not present in line 30, whereas lines 51 and 62 showed patterns that were very similar to that of the negative control plant, but with higher intensity. The integration of the transgene in the genomic DNA was demonstrated by comparing the hybridization of 1.0 μg of undigested DNA of four 1Ax1 expressing lines, and one nontransformed control plant (Figure 5). The hybridization signal in the expressing lines was clearly more intense than that of the negative control.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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Claims

We claim:

1. A method for producing a plant with improved breadmaking characteristics comprising the steps of:

(a) transforming a cell to contain a heterologous DNA segment encoding a protein and derived from a HMW-GS;

(b) growing and maintaining said cell under conditions whereby a transgenic plant is regenerated therefrom;

(c) growing said transgenic plant under conditions whereby said DNA is expressed, whereby the total amount of HMW-GS in cells of said plant is increased.

2. The method of claim 1, further comprising the step of obtaining and growing additional generations of descendants of said transgenic plant which comprise said heterologous DNA segment wherein said heterologous DNA segment is expressed.

3. The method of claim 1 wherein said heterologous DNA segment comprises the 1Ax1 promoter.

4. The method of claim 2 wherein said DNA segment comprises the 1Ax1 promoter.

5. The method of claim 1 wherein said heterologous DNA segment is expressed under the control of the 1Ax1 promoter.

6. The method of claim 2 wherein said heterologous DNA segment is expressed under the control of the 1Ax1 promoter.

7. The method of claim 1 wherein said plant is a wheat plant.

8. The method of claim 2 wherein said plant is a wheat plant.

9. A transgenic plant comprising a heterologous DNA segment encoding a protein, said heterologous DNA segment being under the control of the 1Ax1 promoter.

10. A transgenic plant of claim 9, wherein said plant is a wheat plant.

11. A part of the transgenic plant of claim 9, said part selected from the group consisting of a seed, a pollen, and plant tissue; and wherein said part comprises said heterologous DNA segment.

12. A plant part of claim 11 wherein said part is a seed.

13. Flour made from the seed of claim 12.

14. An edible composition made from the flour of claim 13.

15. Transgenic wheat cells comprising DNA segments encoding at least six different HMW-GS proteins and wherein at least six of said DNA segments are expressed.

16. A transgenic wheat plant comprising the cells of claim 13.

17. A part of the transgenic wheat plant of claim 16, said part selected from the group consisting of a seed, a pollen, and plant tissue; and wherein said part comprises cells of claim 15.

18. A plant part of claim 17 wherein said part is a seed.

19. Flour made from the seed of claim 18.

20. An edible composition made from the flour of claim 19.

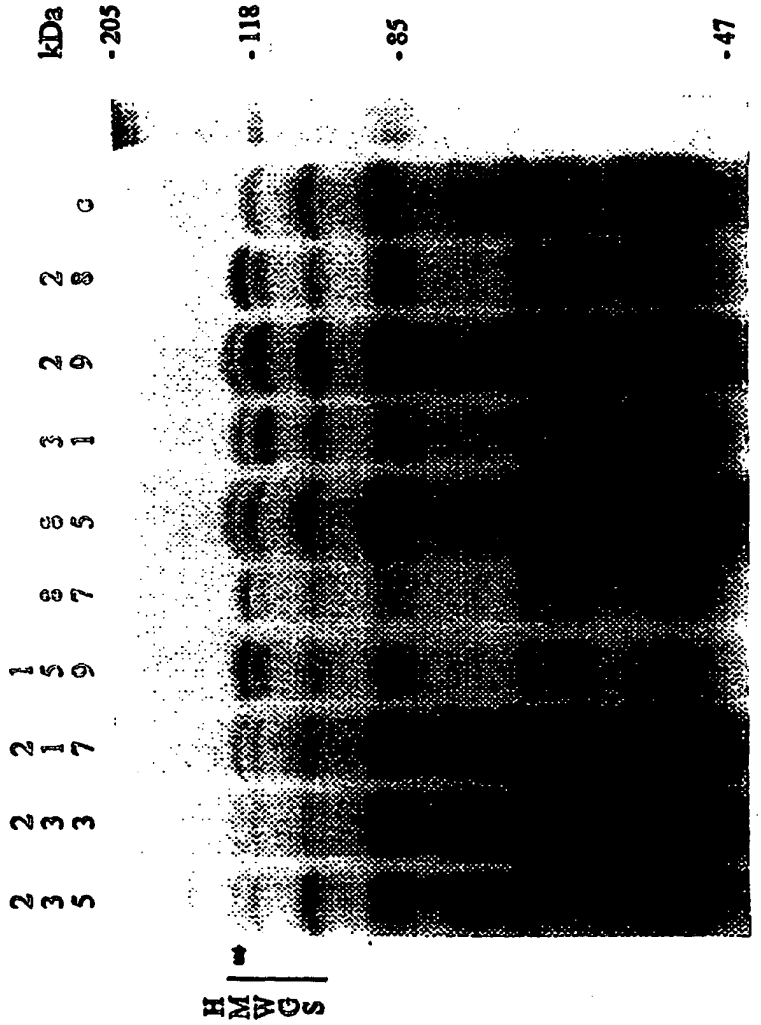

FIG. 1

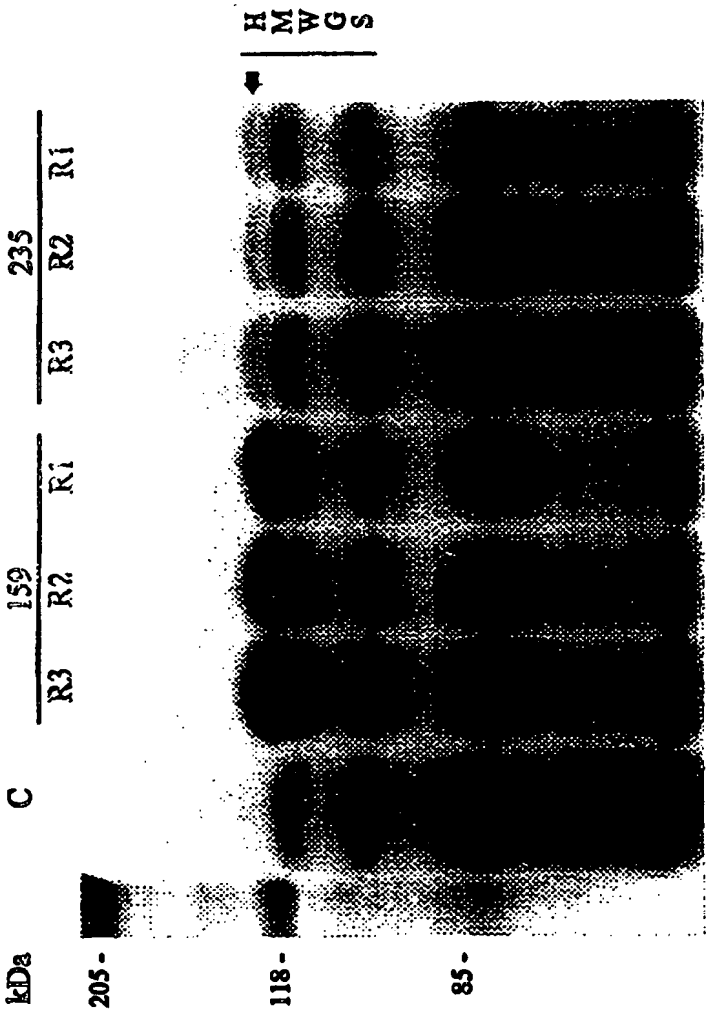

FIG. 2

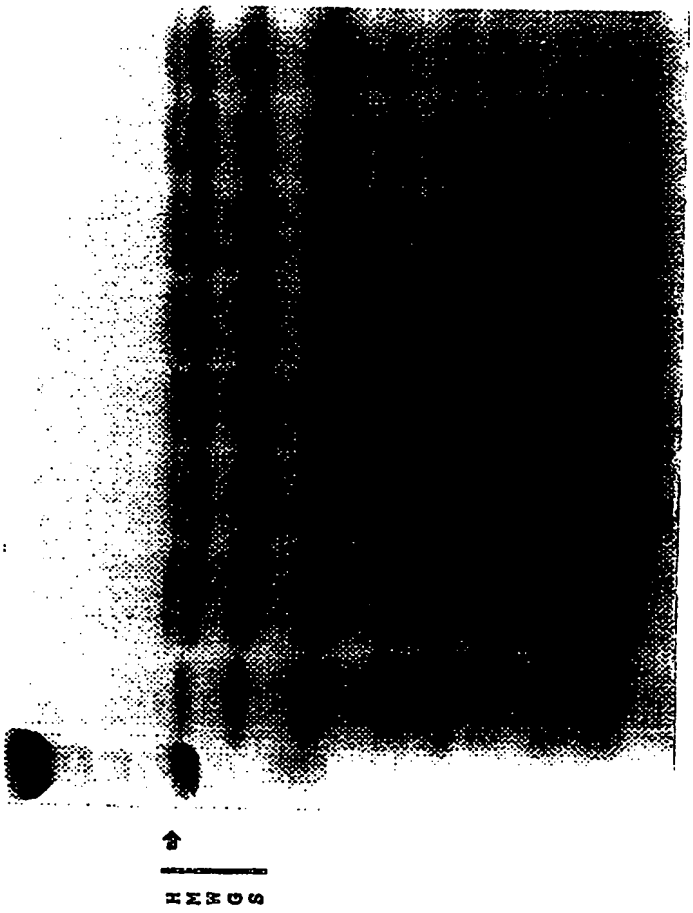

FIG. 3(c)

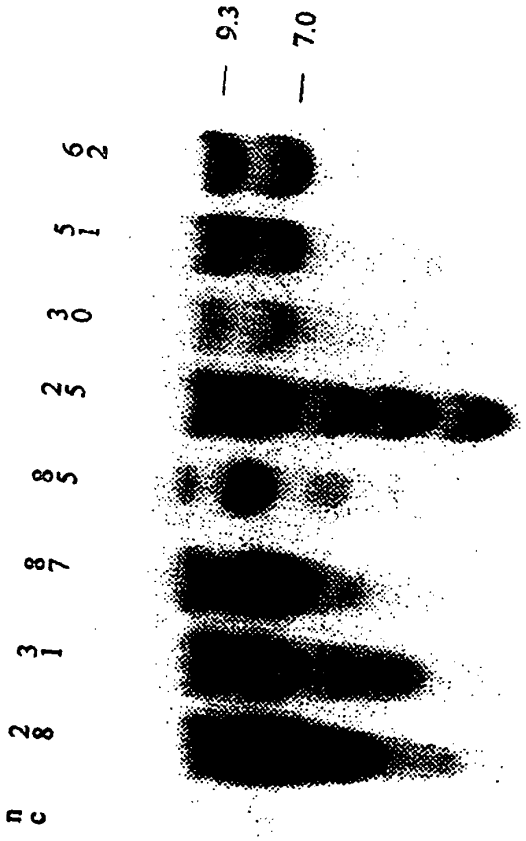

FIG. 4

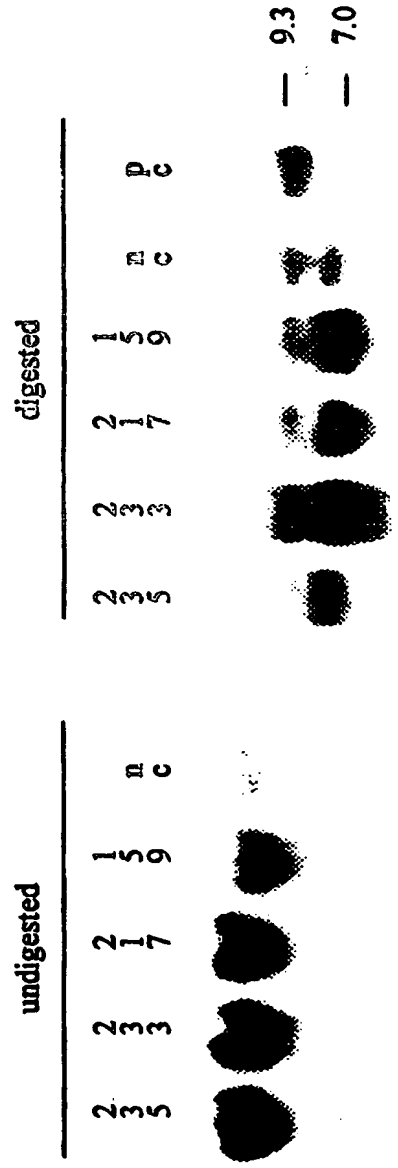

FIG. 5

INTERNATIONAL SEARCH REPORT

Interna .al Application No
PCT/US 97/14534

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/415 C12N15/82 //A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L.S. ROBERT ET AL.: "Tissue-specific expression of a wheat high molecular weight glutenin gene in transgenic tobacco" THE PLANT CELL, vol. 1, no. 6, June 1989, ROCKVILLE US, pages 569-578, XP000035356 see abstract see page 574, column 1, last paragraph ---	1,2
X	A.E. BLECHL ET AL.: "Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat" NATURE BIOTECHNOLOGY., vol. 14, July 1996, UBLISHING US, pages 875-879, XP002049697 cited in the application	1,2
Y	see the whole document --- -/--	3-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1997	09.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

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INTERNATIONAL SEARCH REPORT

Interna .al Application No
PCT/US 97/14534

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P.R. SHEWRY ET AL.: "Biotechnology of bread-making: unraveling and manipulating the multi-protein gluten complex" BIO/TECHNOLOGY., vol. 13, November 1995, NEW YORK US, pages 1185-1190, XP002049698 cited in the application see page 1190, column 2, paragraph 4	15-20
Y	see page 1187, column 1, last paragraph - column 2, paragraph 2 see page 1188, column 2, paragraph 2 - page 1190, column 1, paragraph 3	3-14
A	N.G. HALFORD ET AL.: "Analysis of HMW glutenin subunits encoded by chromosome 1A of bread wheat Triticum-Aestivum L. indicates quantitative effects on grain quality" THEORETICAL AND APPLIED GENETICS, vol. 83, no. 3, 1992, pages 373-378, XP002049699 cited in the application see the whole document	3-14
A	M.S. THOMAS ET AL.: "Identification of an enhancer element for the endosperm-specific expression of high molecular weight glutenin" THE PLANT CELL, vol. 2, no. 12, 1990, ROCKVILLE US, pages 1171-1180, XP002049700 see abstract	3-14
A	N.G. HALFORD ET AL.: "Functional analysis of the upstream regions of a silent and an expressed member of a family of wheat seed protein genes in transgenic tobacco" PLANT SCIENCE , vol. 62, no. 2, 1989, AMSTERDAM NL, pages 207-216, XP002049701 see abstract	3-14
P,X	F. ALTPETER ET AL.: "Integration and expression of the high-molecular-weight glutenin subunit 1Ax1 gene into wheat" NATURE BIOTECHNOLOGY., vol. 14, September 1996, LONDON GB, pages 1155-1159, XP002049702 see the whole document	1-20

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INTERNATIONAL SEARCH REPORT

Interna..al Application No
PCT/US 97/14534

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 25419 A (US GOVERNMENT) 17 July 1997 see page 2 - page 3 see page 6, last paragraph - page 8, paragraph 1 see page 10 - page 14 -----	1,2, 15-20

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/14534

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[illegible]al Application No
PCT/US 97/14534

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725419 A	17-07-97	US 5650558 A	22-07-97
		AU 1698197 A	01-08-97

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